FORM-PTO-1390 (Rev. 10-96) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 003300-679

U.S APPLICATION NO. (If known, see 37 C.F R 1.5): 09/646985

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/SE99/00452	23 March 1999	26 March 1998 & 14 May 1998

TITLE OF INVENTION
PROCESS FOR CONTINUOUS PURIFICATION AND CONCENTRATION OF LEUKOCYTES FROM BLOOD

APPLICANT(S) FOR DO/EO/US
MATS JAREKRANS

It is contemplated that this Application be prosecuted while using Claims 1 to 19 that were submitted on April 19, 2000 during the international phase of prosecution as further amended in the Preliminary Amendment filed herewith.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (executed Declaration will follow)
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

A certified copy of the Swedish Priority Application No. 9801029-1, filed 26 March 1998, was submitted in the International Application. Thus it is believed that the claim for priority has been substantiated.

Small Entity status is claimed.

529 Rec'd PCT/PTO 26 SEP 2000

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/646985		003300-679

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO $840.00 (970)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $670.00 (956)		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $690.00 (958)		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970.00 (960)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 970.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	21 -20 =	1	X$18.00 (966)	$ 18.00	
Independent Claims	2 -3 =	0	X$78.00 (964)	$ --	
Multiple dependent claim(s) (if applicable)			+$260.00 (968)	$ --	
TOTAL OF ABOVE CALCULATIONS =				$ 988.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). Small Entity status is claimed.				$ 494.00	-
SUBTOTAL =				$ 494.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 494.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +				$	
TOTAL FEES ENCLOSED =				$ 494.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 494.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Benton S. Duffett, Jr.
BURNS, DOANE, SWECKER & MATHIS, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404

Benton S. Duffett, Jr.
SIGNATURE

Benton S. Duffett, Jr.
NAME

22,030
REGISTRATION NUMBER

Patent
Attorney's Docket No. 003300-679

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	**BOX PCT**
	)	**Attn: DO/EO/US**
MATS JAREKRANS	)	
	)	
Application No.: Unassigned	)	Group Art Unit: Unassigned
	)	
Filed: September 26, 2000	)	Examiner: Unassigned
	)	
For: PROCESS FOR CONTINUOUS PURIFICATION AND CONCENTRATION OF LEUKOCYTES FROM BLOOD	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This Application corresponds to International Application No. PCT/SE99/00452, filed March 23, 1999.

It is contemplated that this Application be prosecuted in the United States while using Claims 1 to 19 that were filed on April 19, 2000 during the international phase of prosecution as further amended herein.

In the Abstract:

Please add the Abstract of the Disclosure that is provided on a separate sheet.

In the Claims:

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "any of the claims 1-3" and insert --claim 1--.

Claim 6, line 1, delete "any of the claims 1-5" and insert --claim 1--.

Claim 7, line 1, delete "any of the claims 1-6" and insert --claim 1--.

Claim 8, lines 1 and 2, delete "any of the claims 1-7" and insert --claim 1--.

Claim 9, line 1, delete "or 2".

Claim 10, line 1, delete "any of the claims 1-7" and insert --claim 1--.

Claim 11, line 1, delete "any of the preceding claims" and insert --claim 1--.

Please add the following new Claims 20 and 21:

--20. Process according to claim 2, characterized in that in step (b) the aqueous hypotonic solution is ammonium chloride.

21. Process according to claim 2, characterized in that the plasma separated in step (a) is recovered.--

REMARKS

The present Amendment adds an Abstract of the Disclosure on a separate sheet and eliminates the use of multiple dependency.

The examination and allowance of the Application are respectfully requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____

Benton S. Duffett, Jr.
Registration No. 22,030

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: September 26, 2000

Abstract of the Disclosure

Process and apparatus for the continuous purification and concentration of leukocytes from blood, characterized in that said process comprises the following steps: (a) separating plasma from the blood by filtration in order to achieve a filtered buffy coat fraction; (b) adding an aqueous solution, which is hypotonic in relation to plasma, to the buffy coat fraction from step (a), in order to achieve lysation of erythrocytes contained in the buffy coat fraction; (c) mixing the buffy coat fraction and the aqueous hypotonic solution from step (b) in a mixing device; (d) leading the mixture from step (c) through a retention vessel; (e) leading the mixture from step (d) through a centrifuge in order to separate the leukocytes; (f) collecting the separated leukocytes from step (e).

PROCESS FOR CONTINUOUS PURIFICATION AND CONCENTRATION OF LEUKOCYTES FROM BLOOD

Field of the invention

The present invention relates to a new process and apparatus for continuous purification and concentration of leukocytes from blood, preferably buffy coats. The leukocytes are used in the production of interferon.

Prior art

Interferons constitute an endogenously produced immunologically active group of small proteins, which act as a natural defence against viral infections. They are synthesised and secreted by vertebrate cells following a virus infection. Interferons bind to the plasma membrane of other cells in the organism and induce an antiviral state in them by enhancing the production of three enzymes: an oligonucleotide synthetase, an endonuclease, and a kinase. In modern medical care, pharmaceutical compositions containing interferons are administered as a regimen against infections, specially viral infections, but also to generally boost the patient's immunological defence systems.

Interferons are presently manufactured via three different routes: recombinant, cell-line derived and human leukocyte derived. The human leukocyte derived interferon products can further be divided in partially purified and highly purified products. The present application concerns in particular highly purified human leukocyte interferon.

The large scale production of human leukocyte derived interferon is generally performed according to the process outlined by Kari Cantell et al. 1981 (Cantell, K., Hirvonen, S., Kauppinen, H-L. and Myllyla, G., Production of interferon in human leukocytes from normal donors with the use of Sendai virus, in Methods in Enzymol-

ogy, vol 78, p. 29-38, and Cantell, K., Hirvonen, S. and Koistinen, V., Partial purification of human leucocyte interferon on a large scale, in Methods in Enzymology, vol 78, p.499-505.) The process according to Cantell can be summarised as follows: Pooled buffy coats from healthy donors are suspended in cold 0.83 % NH_4Cl and centrifuged. In this step the leukocytes are purified and separated from other blood cells. Approximately 30 % of the leukocytes are lost. The leukocytes are collected and incubated in modified Eagle's minimum essential medium (MEM). Further, the suspension is primed with priming interferon and then inoculated with Sendai virus, to initiate the production of interferon. The harvested crude interferon is then pooled and the interferon precipitated and purified further.

In production with blood an anticoagulant can be added to the blood to prevent clot formation, thereby maintaining the blood in a fluid state. When blood treated in this way is undisturbed, the cells gradually settle because they are denser than the plasma; the red cells go to the bottom, the white cells and platelets form a thin white layer (buffy coat) overlying the red cells, and the plasma appears in the upper portion of the container.

The leukocyte preparation steps are still mostly performed batch wise, using manually handled laboratory flasks and suitable equipment. Scale up has up to now been achieved by adding more flasks and centrifuges and naturally more personnel, handling these flasks. The production of interferon according to the state of the art is thus plagued by the drawbacks, typical for labour intensive processes: high labour costs, low reproducibility, variations in yield etc. Nevertheless, most of the present processes have been focused on how to best utilise available laboratory equipment and methodology.

The present invention aims to overcome these drawbacks and to enable higher yield, better reproducibility

and lower labour costs. Additionally, the invention aims to enable easier scale-up and GMP-verifiability of the process.

Summary of the invention

The present invention offers a solution to the above mentioned problems and shortcomings of conventional processes by introducing a process according to the attached claims. The inventive process has e.g. the advantages of being suitable for automation, thus improving the reproducibility, lowering the operator input needed and reducing labour costs. Further, the inventive process is easy to scale up and adapt to larger production volumes.

Short description of the drawings

The invention will now be described in greater detail below with reference to the accompanying drawings in which

Fig. 1 shows a block diagram showing the principles of a process according to the present invention;

Fig. 2 shows a schematic example of an automated leucocyte purification process according to the present invention.

Description of the invention

The present inventor has surprisingly shown that a continuous and automated process can be used in the purification and concentration of leukocytes. According to the invention there is provided a process for the continuous purification and concentration of leukocytes from blood. The process comprises the following steps:

(a) separating plasma from the blood by filtration in order to achieve a filtered buffy coat fraction;

(b) adding an aqueous solution, which is hypotonic in relation to plasma, to the buffy coat fraction from step (a), in order to achieve lysation of erythrocytes contained in the buffy coat fraction;

(c) mixing the buffy coat fraction and the aqueous hypotonic solution from step (b) in a mixing device;

(d) leading the mixture from step (c) through a retention vessel;

(e) leading the mixture from step (d) through a centrifuge in order to separate the leukocytes ;

(f) collecting the separated leukocytes from step (e).

According to a further aspect of the invention an apparatus is provided for continuous purification and concentration of leukocytes, from blood. The apparatus includes the following means:

(i) a membrane filter means for separating plasma from the blood by filtration in order to achieve a filtered buffy coat fraction;

(ii) a static mixer means for mixing the buffy coat fraction and an aqueous hypotonic solution in order to achieve lysation of erythrocytes contained in the buffy coat fraction;

(iii) a retention vessel means; and

(iv) a centrifuge means in order to separate the leukocytes.

Referring to the block diagram in Fig. 1, the first step in the present process is a step of separating the plasma from blood by means of filtration. According to known processes plasma is usually separated from blood by centrifugation, which can result in a plasma fraction contaminated with lysed erythrocytes, which plasma fraction is of no further use. See e.g U.S. 4,294,824. The use of centrifugation in stead of filtration implies higher investment, more service and higher demands of sanitation and cleaning. In the present invention the plasma is separated from the blood by filtration and then the erythrocytes contained in the buffy coat obtained from the separation is lysed. According to a preferred embodiment of the invention the process starts with a commercially available buffy coat fraction obtained from a blood bank. This buffy coat fraction is separated from

plasma contained therein by filtration. The plasma obtained is pure and can be further used. In the present invention it was surprisingly found that by separating plasma by filtration it was possible to obtain a continuous process that could be scaled up for large scale production. With conventional centrifuge separation in this step it was not possible to scale up the process. The centrifuge step, when scaled up, tended to crush the erythrocytes, thereby contaminating the buffy coat and the plasma. In large scale filtration the erythrocytes remained unchanged in the buffy coat and the separated plasma could be further used.

The filtration parameters in the process are balanced to produce, on one hand, a dense concentrated buffy coat fraction containing the leukocytes and, on the other hand, a pure plasma fraction, with as little discoloration as possible. The plasma fraction can be used as starting material in other processes since it is pure and contains no additives. According to one embodiment of the invention, the filtration step for separation of plasma and leukocytes, is performed with the aid of a membrane filter. Suitable filters have pore sizes within the interval of 0.1 - 1.0 μm, preferably within 0.4 - 0.6 μm. According to another embodiment the filtration is made through hollow fibres, suitable for use in the inventive process and as a part of the inventive apparatus or system.

In order to achieve lysation of the erythrocytes in buffy coat fraction, this fraction is mixed with a hypotonic solution in relation to plasma, e.g. aqueous ammonium chloride solution (e.g. 0.8 - 0 % w/v) or, preferably 0.8 %, but when using lower concentrations the lysation time has to be decreased. The flow of hypotonic solution is preferably twice the flow of the buffy coat fraction. To ensure effective mixing, the mixture of the buffy coat fraction and e.g. the NH_4Cl solution is lead through a static mixer and further to a retention vessel.

The retention vessel is designed in a manner, considering the flow/volume ratio, that a retention time of about 0.5 - 10 minutes is achieved, depending on the kind of hypotonic solution and the temperature used, and that the solution becomes homogenous in the entire vessel. The retention vessel is designated in a manner that a retention time of preferably 5 - 10 minutes is achieved when ammonium chloride solution is used, most preferably 10 minutes if cold 0.8 % ammonium chloride solution is used.

Subsequently, the mixture of the buffy coat fraction and the NH_4Cl solution is supplied to a continuous centrifuge in order to separate the leukocytes. The centrifuge can be a continuous or semi-continuous centrifuge of sanitary design and preferably a centrifuge adapted for sanitation without dismantling, Sanitation-In-Place (SIP) and also Clean-In-Place (CIP). In this step there is no problem to use a centrifuge for the separation, in a large scale process, as the erythrocytes have been removed by lysation.

Further processing may include a second erythrocyte lysis step. Finally, the purified and concentrated leukocytes, also called the cell suspension, are transferred to an incubation vessel, where the interferon production is induced by adding Sendai virus. Preferably the interferon production is carried out in a bioreactor. The advantages with using a bioreactor as the incubation vessel is the possibility to achieve better control of the incubation step, easier scale-up, facilitated Sanitation-In-Place, and Cleaning-In-Place. Together with the other steps described earlier in the text, this will constitute a more complex process better adapted to industrial manufacturing of interferon.

According to a preferred embodiment the blood used in the present invention is human blood.

EXAMPLES

Example 1.

Plastic bags containing the buffy coat fraction are taken out from the ordinary production batch and emptied separately. Half of the amount of this fraction was emptied in a separate vessel (Figure 2., a) and processed through the experimental process according to Figure 2 and the other half according to the ordinary production process. In the experimental process, the plasma is separated by filtration through hollow fibres with appropriate fibre diameter and pore size (b). The filtered plasma is fractionated and the fractions with highest absorbency at 280 nm (A_{280}) are stored for later functional tests (results shown in Table 2.). The collection of plasma with highest absorbency is accomplished by measuring the absorbency at 280 nm on-line by means of a spectrophotometer (c) and when the absorbency has reached a predetermined value the valve (d) is automatically opened in the plasma vessel (e) direction. The spectrophotometer (c) is also utilised for measuring the amount of disrupted erythrocytes in the plasma. When erythrocytes are disrupted by e.g. mechanical forces they are lysed and the plasma becomes reddish due to the free haemoglobin. If the filtered plasma, for some reason becomes reddish it is discarded by setting a second criteria for the spectrophotometer (c) at about 410 nm. When the absorbency has reached a predetermined value at 410 nm, the valve (d) is automatically opened in the waste (f) direction.

Mechanical forces can, as mentioned earlier disrupt the erythrocytes. One way to disrupt the erythrocytes is to have to high feed pressure into the hollow fibres. Therefore, the feed pressure is automatically regulated by measuring the feed pressure with a pressure sensor (g) and to automatically adjust the pump (h) speed with respect to the feed pressure in order to keep the feed pressure constant at about 0,4 bars.

After the separation of plasma, the leukocyte fraction flows directly into a mixing chamber, a static mixer together with ammonium chloride. The flow of the leukocyte fraction is measured by a first flow meter (i). The flow of 0,8% (w/v) ammonium chloride fraction is measured by a second flow meter (j). Since the leukocyte fraction flow can change during the filtration step the flow of 0,8% (w/v) ammonium chloride fraction is automatically regulated with respect to the leukocyte fraction flow. The ammonium chloride flow is twice the leukocyte fraction flow. The blended solution is then entered into a static mixer (k) in order obtain a complete mixing of the two solutions. The mixed solution flows then continuously into the retention vessel (l). The retention time in the retention vessel is ten minutes which is the time for lysation according to the ordinary batch record. The lysate, which comes out from the retention vessel flows directly into the semi-continous centrifuge (m), which was run at 3000 rpm. The centrifuge was then intermittently harvested. The harvested cells were lysed once more and suspended with some medium to form the cell suspension.

Certain amounts of the cell suspension are then added to 100 ml incubation flasks or to 3 litre laboratory fermentors in order to compare cells from the ordinary production process and the cells from the experimental process. The interferon from the labfermentors and 100 ml flasks are then analysed for interferon content by an ELISA method.

In the ordinary process the fractionation of plasma is achieved by centrifugation of the buffy coat fraction. The centrifugation is performed in one litre plastic bottles. The lysis occurs in a vessel, where the leukocyte fraction and the 0,8% (w/v) ammonium chloride solution are mixed and left to stand for ten minutes. The following centrifugation step is also carried out in one litre plastic bottles.

Table 1. Experimental yields: interferon

	Amount add. (ml)	Cellconc. (x 107 st/ml)	ELISA (IU/ml)	Yield/cell (10-3 IU/cell)	Yield (%)	Yield/cell (%)	Tot. yield.
961009							
Exp.-cells	1,5	0,8	32000	4	100	100	100%
Ref.-cells	1,5	0,8	32000	4			
961023							
Exp.-cells	1,1	1	41000	4,1			
Exp.-cells	1,1	1	47000	4,7	85	90	155%
Ref.-cells	2	1	52000	5,2			
Ref.-cells	2	1,1	51000	4,6			
961030							
Exp.-cells	1	0,8	42000	5,3			
Exp.-cells	1	0,8	43000	5,4			
Ref.-cells	1	0,8	46000	5,8			
Ref.-cells	1	0,8	46000	5,8			
Exp.cells	1,5	1,1	61000	5,5			
Exp.-cells	1,5	1,1	54000	4,9	91	91	91%
Ref.-cells	1,5	1,1	65000	5,9			
Ref.-cells	1,5	1,1	64000	5,8			
961114							
Exp.- cells	1	1,1	38000	3,5			
Exp.- cells	1	1,1	37000	3,4			
Exp.- cells	1,5	1,7	56000	3,3			
Exp.- cells	1,5	1,7	56000	3,3	84	82	84%
Ref.-cells	1	1,1	42000	3,8			
Ref.-cells	1	1,1	46000	4,2			
Ref.-cells	1,5	1,6	70000	4,4			
Ref.-cells	1,5	1,6	64000	4			
961121							
Exp.- cells	1,5	1,1	58000	5,3			
Exp.- cells	1,5	1,1	53000	4,8			
Exp.- cells	2	1,5	73000	4,9			
Exp.- cells	2	1,5	66000	4,4	139	94	196%
Ref.-cells	2	0,7	29000	4,1			
Ref.-cells	2	0,7	46000	6,9			
Ref.-cells	3	1,1	56000	5,1			
Ref.-cells	3	1,1	51000	4,6			
961127							
Exp.- cells	1,1	1,1	41000	3,7			
Exp.- cells	1,1	1,1	47000	4,3			
Exp- cells	1,5	1,7	69000	4,1			
Exp.- cells	1,5	1,7	69000	4,1	128	92	193%
Ref.-cells	1,5	0,8	32000	4			
Ref.-cells	1,5	0,8	36000	4,5			
Ref.-cells	2,5	1,2	53000	4,4			
Ref.-cells	2,5	1,2	56000	4,7			
LabfermExp	45	0,8	46000	5,8			
LabfermRef	84	0,8	40000	5			

961204							
Exp.- cells	1	0,8	30000	3,8			
Exp.- cells	1	0,8	31000	3,9			
Exp.- cells	1,5	1,2	48000	4			
Exp.- cells	1,5	1,2	50000	4,2	87	96	87%
Ref.-cells	1	0,9	37000	4,1			
Ref.-cells	1	0,9	35000	3,9			
Ref.-cells	1,5	1,4	53000	4,4			
Ref.-cells	1,5	1,4	56000	4,1			
Labferm Exp	65	1,1	50000	4,5			
Labferm Ref	65	1,1	55000	5			
961206							
Exp.- cells	1,5	1	51000	5,1			
Exp.- cells	1,5	1	48000	4,8			
Exp.- cells	2,25	1,5	83000	5,5			
Exp.- cells	2,25	1,5	79000	5,3	116	116	78%
Ref.-cells	1	1	40000	4			
Ref.-cells	1	1	50000	5			
Ref.-cells	1,5	1,5	70000	4,7			
Ref.-cells	1,5	1,5	62000	4,1			
961211							
Exp.- cells	1,5	1,1	66000	6			
Exp.- cells	1,5	1,1	61000	5,5			
Exp.- cells	2	1,4	88000	6,3			
Exp.- cells	2	1,4	86000	6,1	106	89	106%
Ref.-cells	1,5	0,9	61000	6,8			
Ref.-cells	1,5	0,9	67000	7,4			
Ref.-cells	2	1,2	79000	6,6			
Ref.-cells	2	1,2	74000	6,2			
Labferm.Exp	65	1	68000	6,8			
Labferm.Ref	65	1	64000	6,4			
MEAN					104	94	121
STAND. DEV.					20	10	47
C.V.					19%	10%	39%
NO.					9	9	9

Table 1

The Table shows the result from functional tests of cells from the process according to the invention in comparison to cells from an ordinary batch. Exp.-cells means cells obtained from the process according to the invention. Ref.-cells means cells from an ordinary batch prepared from buffy coats from the same blood centre and the same day. LabfermExp means cells obtained from the process according to the invention but the test is performed in larger scale (3 litre) than Exp.-cells. LabfermRef means cells from an ordinary batch prepared from buffy coats from the same blood centre and the same day invention but the test is performed in larger scale (3 litre) than Ref.-cells. Amount add. means that different amounts of cell suspension have been added in order to achieve different cell concentrations for the functional test.. Cellconc. is the measured cell concentration after addition of the amount of cells. ELISA is the result expressed in the amount of intereferon-alpha produced by the different cells per millilitre. Yield/cell is the result expressed in the amount of intereferon-alpha produced by the different cells per cell. Yield is the average result in percentage expressed in the amount of intereferon-alpha produced per millilitre by the Exp.cells/LabfermExp in comparison with the Ref.-cells/LabfermRef. Yield/cell II is the average result in percentage expressed in the amount of intereferon-alpha produced per cell by the Exp.cells/LabfermExp in comparison with the Ref.-cells/LabfermRef. Tot. yield is the total average amount of produced interferon-alpha by the Exp.cells/LabfermExp if the same amount of cells have been added. in percentage in comparison with the Ref.-cells/LabfermRef.

The results in Table 1 shows in average a better total yield with the inventive, Exp. cells in comparison with Ref.-cells. Since the two processes, Exp.-cell and Ref.-cell started with the same amount of cells and the

volume of the final concentrated leukocyte cell suspension is the same for both processes, it is possible to calculate the recovery of cells from each process. get more cells from the exp. When comparing the cell concentration in the trials performed 961121 and 961127 when the same amount of cell suspension have been added, it is obvious that the inventive process results in a higher cell recovery. Therefore, it is also possible to get more interferon from the inventive process since the yield and yield per cell is about the same.

The plasma recovered from the filtration step is used as a component in the incubation medium (EMEM). Before the plasma is added to the incubation medium, the immunoglobulin fraction is precipitated through addition of 25% (w/w) polyethylene-glycol (Macrogol 6000) solution. The polyethylene-glycol solution is prepared by solving 430 g into one litre distilled water.

In the ordinary process, an extra centrifugation step has to be performed (1600 rpm) on the plasma fraction in order to remove remaining cells.

The functional tests for plasma separated by hollow fibres was performed through adding this plasma instead of the ordinary plasma to the medium when incubating leukocytes in small scale, 100 ml flasks. Plasma was added to the incubation medium in equal amounts with respect to protein content, A_{280} value. The interferon produced in these flasks were analysed by the ELISA-method and compared with ELISA-results from flasks ran with ordinary plasma.

Table 2. Experimental yields: plasma quality

Plasma prep.	A 280	Amount add. (ml)	Media add. (ml)	Cellconc. (x 107 st/ml)	ELISA (IU/ml)	Yield/cell (10-3 IU/cell)	Yield/cell (%)	Yield (%)
M-5296, Ref.	25,6	1,6	40	1,2	63000	5,3	100	100
M-5296, Ref.	25,6	1,6	40	1,2	62000	5,2	100	100
961014	18,4	2,23	39,4	1,2	71000	5,9	115	115
961014	18,4	2,23	39,4	1,2	73000	6,1		
961028	17,3	2,38	39,2	1,2	64000	5,3	109	109
961028	17,3	2,38	39,2	1,2	72000	6,0		
961030	18,8	2,25	39,4	1,2	65000	5,4	102	102
961030	18,8	2,25	39,4	1,2	62000	5,2		
961114	14,6	2,81	38,8	1,2	66000	5,5	102	102
961114	14,6	2,81	38,8	1,2	62000	5,2		
961202	12,3	3,28	38,3	1,2	64000	5,3	106	106
961202	12,3	3,28	38,3	1,2	68000	5,7		
MEAN							107	107
STAND. DEV.							6	6
C.V.							5%	5%
NO.							5	5

Table 2.

The Table shows the result from functional tests of plasmas from the process according to the invention in comparison to plasma from an ordinary batch, M-5296, Ref . 961014, 961028, 961030, 961104 and 961202 are the dates when the filtered plasma were prepared. A 280 is the absorbency at 280 nm. Amount add. means that different amounts of plasma have been added in order to achieve the same protein content in the incubation medium. Media add. means the amount of incubation media used. Cellconc. is the measured cell concentration after addition of cells from the same cell suspension. ELISA is the result expressed in the amount of intereferon-alpha produced per millilitre. Yield/cell is the result expressed in the amount of intereferon-alpha per cell. Yield is the average result in percentage expressed in the amount of intereferon-alpha produced per millilitre by the cells incubated with different plasma prepared according to the inventive process in comparison with the plasma from an ordinary batch, M-5296. Yield/cell II is the average result in percentage expressed in the amount of intereferon-alpha produced per cell by the different plasma preparations in comparison with. Tot. yield is the total average amount of produced interferon-alpha by the cells incubated with different plasma preparations in percentage in comparison with the M-5296, Ref.

Example 2.

Sanitation of the inventive process system was performed stepwise automatically:

Flushing of the buffy coat vessel (a) and filters (b) with PBS (phosphate buffered saline) from vessel (n).

Reversing the flow direction of PBS in the plasma filter

Flushing of the static mixer (k), retention vessel (l) and centrifuge (m) with NH_4Cl from vessel (o).

Drainage of the whole system.

Cleaning of the whole system with 0,5 M NaOH from vessel (p) for at least half an hour.

Drainage of the whole system.

The system is then filled with storage solution, 25% ethanol (v/v) from vessel (q).

Before use, the system is drained and then flushed with distilled water and then sterile PBS.

After sanitation, the system was disassembled at critical points and inspected visually to see that no cells, cell fragments etc. have been retained inside the system. Samples were taken out on sterile PBS or on sterile water before each trial when the solution has passed the whole system or as indicated in table 3. The samples were analysed for bacterial counting (according to the European Pharmacopeia, 2nd Edition) and endotoxin determination (according to the European Pharmacopeia, 2nd Edition).

Table 3. Experimental results: sanitary status

	Bact. (cfu/ml)	Endotox. (EU/ml)
961009 Wash sol.:PBS	< 0.5	< 0.2
961023 Wash sol.:water	0,5	< 0.2
961030 Wash sol.:water	< 0,5	< 0,2
961114 Wash sol.:water, after hollow fibre Wash sol.:water, after retention vessel	< 0,5 1	< 0,2
961121 Wash sol.:water, filtrate Wash sol.:water, after retention vessel	< 0,5 < 0,5	ND
961127 Wash sol.:water	< 0.5	< 0,2
961204 Wash sol.:water	< 0,5	ND
961206 Wash sol.:water	< 0,5	ND
961211 Wash sol.:water	< 0,5	ND

No cells, cell fragments etc. were seen inside the system. Table 3 shows that the inventive system can be run under aseptic conditions and is suitable for CIP, that is cleaned in place.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

PCT/SE 99/00452
19 -04- 2000

CLAIMS

1. Process for the continuous purification and concentration of leukocytes from blood, characterized in that said process comprises the following steps:

(a) separating plasma from the blood by filtration in order to achieve a filtered buffy coat fraction;

(b) adding an aqueous solution, which is hypotonic in relation to plasma, to the buffy coat fraction from step (a), in order to achieve lysation of erythrocytes contained in the buffy coat fraction;

(c) mixing the buffy coat fraction and the aqueous hypotonic solution from step (b) in a mixing device;

(d) leading the mixture from step (c) through a retention vessel;

(e) leading the mixture from step (d) through a centrifuge in order to separate the leukocytes ;

(f) collecting the separated leukocytes from step (e).

2. Process according to claim 1, characterized in that a buffy coat fraction, obtained from blood, is used in stead of blood in step (a) and plasma is removed from this buffy coat fraction by filtration.

3. Process according to claim 1 or 2, characterized in that in step (b) the aqueous hypotonic solution is ammonium chloride.

4. Process according to any of the claims 1 - 3, characterized in that the filtration is performed by leading the blood through a membrane filter with a pore size in the interval of 0.1 - 1.0 µm.

5. Process according to claim 4, characterized in that the filtration is performed by leading the blood through a membrane filter with a pore size in the interval of 0.4 - 0.6 µm.

6. Process according to any of the claims 1 - 5, characterized in that the retention vessel is designed in a way resulting in a retention time for the mixture in step (d) of about 0.5 - 10 minutes.

7. Process according to any of the claims 1 - 6, characterized in that the leukocytes collected in step (f) are subjected to a second lysis step.

8. Process according to claim any of the claims 1 - 7, characterized in that the leukocytes collected in step (f) are incubated in a bioreactor for interferon production.

9. Process according to claim 1 or 2, characterized in that the plasma separated in step (a) is recovered.

10. Process according to any of the claims 1 - 7, characterized in that the process is automatically operated and adapted for clean in place (CIP) cleaning and (SIP) sanitation in place.

11. Process according to any of the preceding claims, characterized in that the blood is human blood.

12. Apparatus for continuous purification and concentration of leukocytes, from blood, characterized in that said apparatus includes the following means:

(i) a membrane filter means for separating plasma from the blood by filtration in order to achieve a filtered buffy coat fraction;

(ii) a static mixer means connected to said membrane filter means and receiving said buffy coat fraction for mixing the buffy coat fraction and an aqueous hypotonic solution in order to achieve lysation of erythrocytes contained in the buffy coat fraction of the mixture;

(iii) a retention vessel means, connected to said static mixer means for receiving the mixture therefrom and designed in a way for the mixture to become homogeneous;

(iv) a centrifuge means connected to said retention vessel means and arranged to separate the leukocytes from the mixture from the retention vessel.

13. Apparatus according to claim 12, characterized in that, a buffy coat fraction, obtained from blood, is used in stead of blood and plasma is removed from this buffy coat fraction by filtration.

14. Apparatus according to claim 12, characterized in that the aqueous hypotonic solution is ammonium chloride.

15. Apparatus according to claim 12, characterized in that the membrane filter means is a filter with a pore size in the interval of 0.1 - 1.0 µm.

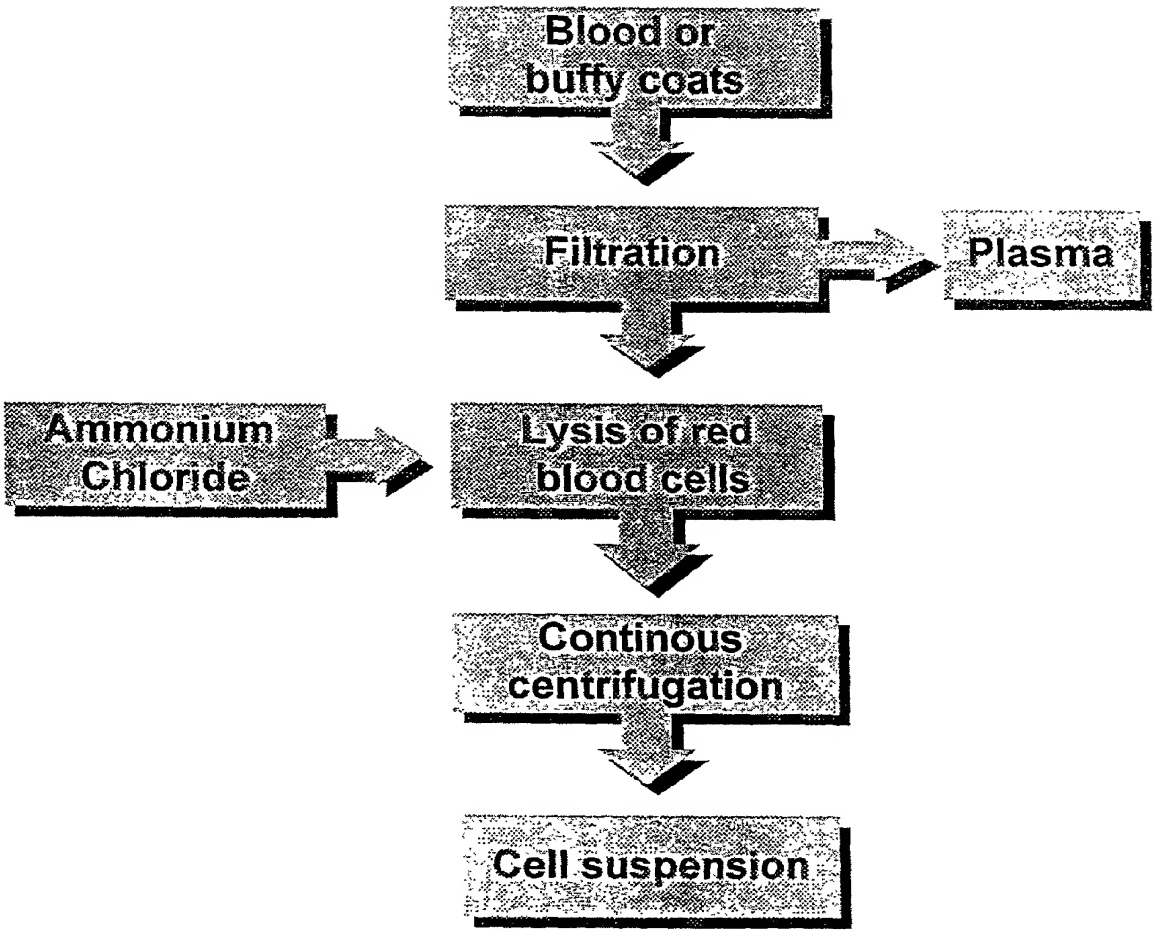
16. Apparatus according to claim 14, characterized in that the membrane filter means is a filter with a pore size in the interval of 0.4 - 0.6 µm.

17. Apparatus according to claim 12, characterized in that the retention vessel means is designed in a way resulting in a retention time for the mixture in the retention vessel of about 0.5 - 10 minutes.

18. Apparatus according to claim 12, characterized in that the centrifuge is adapted to continuous or semi-continuous separation of the leukocytes.

19. Apparatus according to claim 12, characterized in that said apparatus is equipped for cleaning and sanitation, which cleaning and sanitation does not require the dismantling of the equipment, so called clean in place (CIP) and sanitation in place (SIP).

Fig. 1



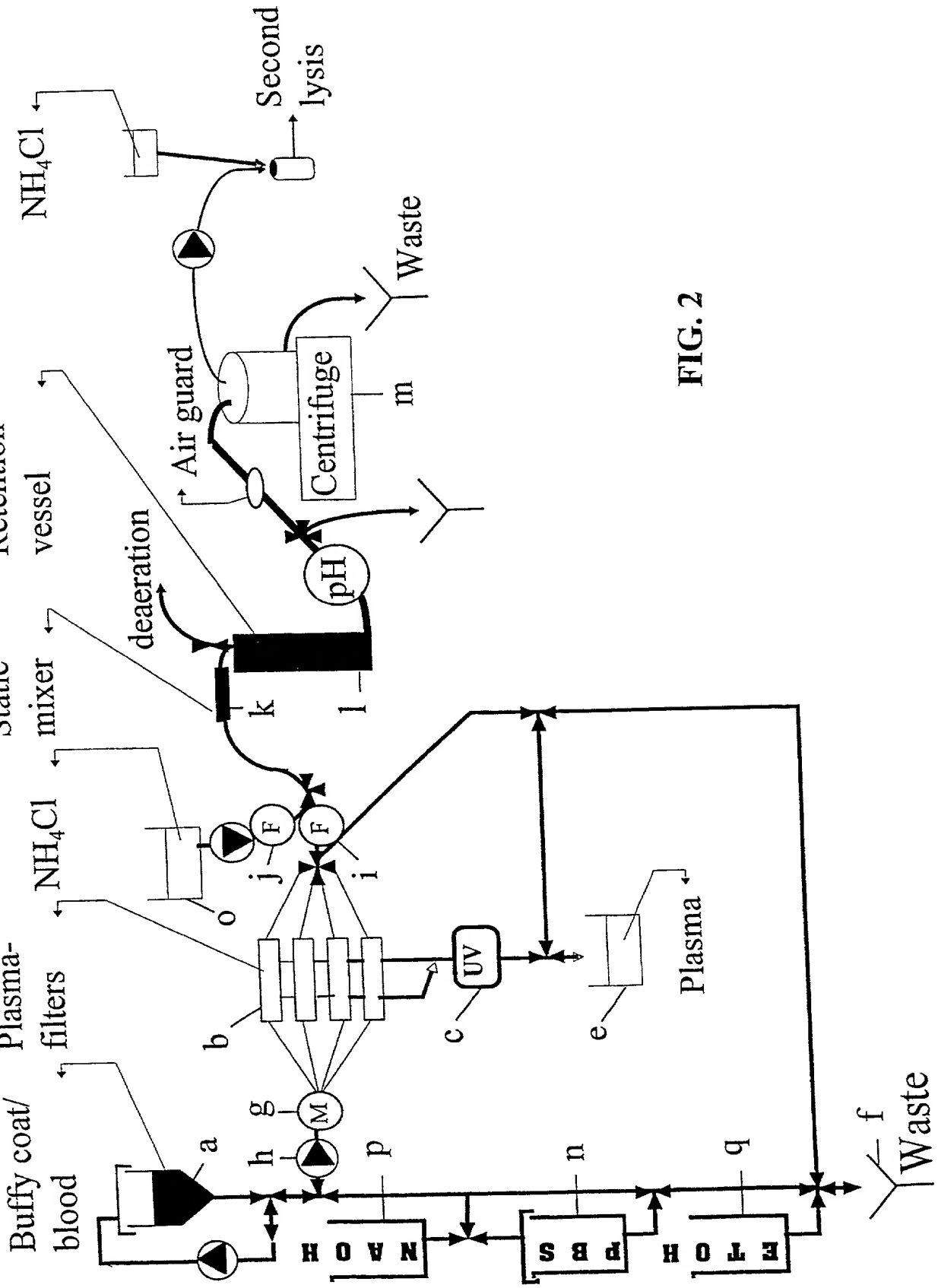


FIG. 2

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 003300-679

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name;
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESS FOR CONTINUOUS PURIFICATION AND CONCENTRATION OF LEUKOCYTES FROM BLOOD

the specification of which (check only one item below):

☒ is attached hereto.

☐ was filed as United States application
Number _____
on _____
and was amended
on _____ (if applicable).

☐ was filed as PCT international application
Number _____
on _____
and was amended
on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(e) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. §119
Sweden	9801029-1	26 March 1998	X Yes _ No
			_ Yes _ No
			_ Yes _ No
			_ Yes _ No
			_ Yes _ No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

60/085,391	14 May 1998
(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

(5/00)

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 003300-679

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to the patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120:

U.S. APPLICATIONS		STATUS *(check one)*		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NUMBERS ASSIGNED (if any)			
PCT/SE99/00452	23 March 1999			X	

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

William L. Mathis	17,337	R. Danny Huntington	27,903	Gerald F. Swiss	30,113
Robert S. Swecker	19,885	Eric H. Weisblatt	30,505	Charles F. Wieland III	33,096
Platon N. Mandros	22,124	James W. Peterson	26,057	Bruce T. Wieder	33,815
Benton S. Duffett, Jr.	22,030	Teresa Stanek Rea	30,427	Todd R. Walters	34,040
Norman H. Stepno	22,716	Robert E. Krebs	25,885	Ronni S. Jillions	31,979
Ronald L. Grudziecki	24,970	William C. Rowland	30,888	Harold R. Brown III	36,341
Frederick G. Michaud, Jr.	26,003	T. Gene Dillahunty	25,423	Allen R. Baum	36,086
Alan E. Kopecki	25,813	Patrick C. Keane	32,858	Steven M. du Bois	35,023
Regis E. Slutter	26,999	Bruce J. Boggs, Jr.	32,344	Brian P. O'Shaughnessy	32,747
Samuel C. Miller, III	27,360	William H. Benz	25,952	Kenneth B. Leffler	36,075
Robert G. Mukai	28,531	Peter K. Skiff	31,917	Fred W. Hathaway	32,236
George A. Hovanec, Jr.	28,223	Richard J. McGrath	29,195		
James A. LaBarre	28,632	Matthew L. Schneider	32,814		
E. Joseph Gess	28,510	Michael G. Savage	32,596		

21839

and: none

Address all correspondence to:

21839

Benton S. Duffett, Jr.
BURNS, DOANE, SWECKER & MATHIS, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404

Address all telephone calls to: Benton S. Duffett, Jr. at (703) 836-6620.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 003300-679

FULL NAME OF SOLE OR FIRST INVENTOR MATS JAREKRANS	SIGNATURE X Mats Jarekrans	DATE X 2000-11-01
RESIDENCE Umeå, Sweden SEX		CITIZENSHIP Sweden
POST OFFICE ADDRESS Trastvägen 19B, SE 907 19, Umeå, Sweden		
FULL NAME OF SECOND JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF THIRD JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF EIGHTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		